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Respirometric Multi-Level Biodegradation Testing Protocol and Methodology for Determining Biodegradation Kinetic Data

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INTRODUCTION

Electrolytic respirometry is attaining prominence in biodegradation studies and is becoming one of the more suitable experimental methods for measuring the biodegradability and the kinetics of biodegradation of toxic organic compounds by the sewage, sludge and soil microbiota and for determining substrate inhibitory effects to microorganisms in wastewater treatment systems.

Biodegradation of toxic and hazardous organic compounds holds a great promise as an important fate mechanism in wastewater treatment and in soil detoxification. Information about the extent and rate of biodegradation is a prerequisite for informed decision making on the applicability of the biodegradation approach. Unfortunately, relatively little quantitative data are available from which engineering judgement can be made, because of the large effort required to assess biodegradation kinetics.

Current research in our laboratories has shown that it is possible to assess biodegradation kinetic parameters from oxygen uptake data, obtained through the use of electrolytic respirometry. This method greatly reduces the work and expense involved in evaluation of biodegradation kinetics.

The objectives of the program is to utilize the electrolytic respirometry oxygen uptake data to: (1) determine the biodegradability of selected RCRA alkyl, chloro, and nitrobenzenes, phenols, phthalates, and ketones and representative CERCLA leachate toxic organics; (2) generate information on their acclimated times (t_0) and the initiation and termination time values for the declining growth phase (t_1 and t_2); (3) determine their first order kinetic parameters of biodegradation (specific growth rate constants for the exponential growth phase (μ) and for the declining growth phase (μ'); (4) estimate the Monod kinetic parameters (μ_m, K_s and Y) of these compounds without initial growth or growth yield assumptions; (5) demonstrate presence of any inhibitory effects of these compounds on the metabolism of the biodegradable reference compound, aniline; and (6) to correlate the extent and rate of biodegradation of these compounds with a predictive model based on chemical properties and structure of these compounds.

BACKGROUND

Use of Electrolytic Respirometry to Generate Biokinetic Data

Dojlido,[1] Larson and Perry,[2] Tabak *et al.*,[3] Oshima *et al.*,[4] Gaudy *et al.*,[5,6] Grady *et al.*[7-9] investigated the use of respirometry to generate biodegradation kinetic data. Larson and Perry[2] showed that the electrolytic respirometer can be used to measure biodegradation of complex organics in natural waters when specific analytical methods or radiolabeled materials are unavailable. However, they used empirical kinetic expressions which were system specific.

Dojlido[1] divided the oxygen uptake curve into seven different phases and then proposed an empirical model for each phase and evaluated the biodegradability and toxicity of a test compound by measuring empirical rate constants and time interval associated with each phase. Various phases were distinguished by identifying inflection points in the curve through the plots of the logarithm of the slope versus time.

Tabak *et al.*[3] and Oshima *et al.*[4] sought to capitalize on Dojlido's method of identifying inflection points in order to quantify more fundamental rate coefficients. In order to identify more fundamental (and therefore intrinsic) kinetic coefficients they used the generalized concept of oxygen uptake by Gaudy and Gaudy.[10] Substrate removal was divided into exponential and declining phases separated by an inflection point, and the endogenous phase. They coupled substrate removal and cell growth to oxygen consumption by imposition of an electron balance and consequently were able to evaluate μ_m from oxygen consumption data up to the inflection point. They proposed the use of the lag time as an indicator of how difficult it is to achieve acclimation to a test compound and their respirometric studies were carried out with an unacclimated biomass.

The justification for using respirometry to obtain intrinsic kinetic lies in the concept of oxygen consumption as an energy balance (Busch *et al.*[11]; Gaudy and Gaudy[10]). This concept states that all of the electrons available in a substrate undergoing biodegradation must either be transferred to the terminal acceptor or be incorporated into new biomass or soluble microbial products. If the concentrations of the substrate, the products and the biomass are all expressed in units of chemical demand (COD), then the oxygen uptake can be calculated in a batch reactor from an equation relating oxygen uptake to substrate, biomass and soluble products (Grady *et al.*[7]). Furthermore, since biomass growth and product formation are proportional to substrate removal, this suggests that an oxygen uptake curve can provide the same information as either a substrate removal curve or a biomass growth curve. This latter concept has recently been used by Gaudy *et al.*[5,6] to calculate biodegradation kinetics. Specific growth rates obtained from growth studies as slopes of plots of Ln (biomass concentration) or Ln(X) versus time at different substrate concentrations, compared favorably with those obtained from exponential phase of respirometric oxygen uptake curves as slopes of plots of Ln (d oxygen uptake)/d time or Ln (dO_u/dt) versus time.

Studies of Grady *et al.*[7,8] have demonstrated that it is possible to determine intrinsic kinetics of single organic compounds by using only measurements of oxygen consumption in respirometric batch reactors. With the use of computer simulation techniques and non-linear curve fitting methods, intrinsic kinetic parameters were obtained from oxygen consumption data and were shown to be in agreement with those obtained from traditional measurement of substrate removal (DOC, SCOD, ^{14}C) or cell growth.

Experimental Approach

The electrolytic respirometry studies were conducted using an automated continuous oxygen uptake and BOD measuring Voith Sapromat B-12 (12 unit system) electrolytic respirometer-analyzer. The instrument consists of a temperature controlled waterbath, containing measuring units, a recorder for digital indication and direct plotting of the decomposition velocity curves of organic compounds; and a cooling unit for the conditioning and continuous recirculation of waterbath volume. The recorder shows the digital indication of oxygen uptake and constructs a graph for these values of each measuring unit. The cooling unit constantly recirculates water to maintain constant temperature in the waterbath. Each measuring unit is comprised of a reaction vessel with a carbon dioxide absorber mounted in a glass joint flask stopper, an oxygen generator and a pressure indicator. This measuring unit is interconnected by hoses, forming an air sealed system, so that the atmospheric pressure fluctuations do not adversely affect the results.

The activity of the microorganisms in the sample creates a vacuum which is recorded by the pressure indicator, which triggers the oxygen generator. The pressure conditions are balanced by electrolytic oxygen generation. The quantity of the sample, the amperage for the electrolysis and the speed of the synchronous motor are so adjusted that, with a sample of 250 mL, the digital counter indicates the oxygen uptake directly in mg/L. The CO_2 generated is absorbed by soda lime. The nitrogen/oxygen ratio in the gas phase above the sample is maintained throughout the experiment and there is no depletion of oxygen. The recorder-plotter concomitantly constructs an oxygen uptake graph for the selected values. The oxygen generators of the individual measuring units are electrolytic cells which supply the required amount of oxygen by electrolytic dissociation of a copper sulfate solution combined with sulfuric acid.

The nutrient solution used in these studies was an OECD synthetic medium[12,13] consisting of measured amounts per liter of deionized distilled water of (1) mineral salts solution; (2) trace salts solution, and (3) a solution (150 mg/L) of yeast extract as a substitute for vitamin solution. The microbial inoculum was an activated sludge from The Little Miami wastewater treatment plant in Cincinnati, Ohio, receiving municipal wastewater. The activated sludge sample was aerated for 24 hours before use to bring it to an endogenous phase. The sludge biomass was added to the medium at a concentration of 30 mg/L total solids. Total volumes of the synthetic medium in the 500 mL capacity reactor vessels were brought up to a final volume of 250 mL.

The test and control compound concentrations in the media were 100 mg/L. Aniline was used as the biodegradable reference compound, at a concentration of 100 mg/L.

The typical experimental system consisted of duplicate flasks for the reference substance, aniline, and the test compounds, a single flask for the physical/chemical test (compound control), a single flask for toxicity control (test compound plus aniline at 100 mg/L each) and an inoculum control. The contents of the reaction vessels were preliminarily stirred for an hour to ensure endogenous respiration state at the initiation of oxygen uptake measurements. Then the test compounds and aniline were added to it. The reaction vessels were then incubated at 25°C in the dark (enclosed in the temperature controlled waterbath) and stirred continuously throughout the run. The microbiota of the activated sludge used as an inoculum were not pre-acclimated to the substrates. The incubation period of the experimental run was between 28 to 50 days. A more comprehensive description of the procedural steps of the respirometric tests is presented elsewhere.[3,13,14]

For fully automatic data acquisition, frequent recording and storage of large numbers of oxygen uptake data, the Sapromat B-12 recorders are interfaced to an IBM-AT computer via the Metrabyte interface system. The use of Laboratory Handbook software package allows the collection of data at 15 minute intervals.

The oxygen utilization by the biomass based on the oxygen uptake velocity (BOD) curves, consisting of the exponential and declining phases of microbial growth was the basis for measurement of substrate utilization and growth rate.

Chemical Analysis

Indirect analysis of culture samples from respirometric vessels during the study included determination of chemical oxygen demand as soluble and total COD and of dissolved oxidizable carbon (DOC) with the use of a Beckman Model 915 B system.

Specific substrate analysis of culture samples for the residual parent compound and the possible intermediate and final products of metabolism was performed with the use of a Gas Chromatograph Varian 3700, equipped with FID and EC detectors and with different detector columns (depending on the substrate to be analyzed) and the Finnigan automated GC/EI-CI Mass Spectrophotometer system. Samples were analyzed as dichloromethane extracts of cultures (continuous liquid-liquid extraction) or by direct aqueous injection.

Suspended solids and biomass were measured gravimetrically (solids retained on a 0.45 μm filter) and by optical density determined spectrophotometrically (percent transmission read at 540 nm).

Determination of Substrate Biodegradability from Oxygen Uptake Data

In this study, biodegradation was measured by three approaches: the first, as the ratio of the measured BOD values in mg/L (oxygen uptake values of test compound minus inoculum control - endogenous oxygen uptake values) to the theoretical oxygen demand (ThOD) of substrate as a percent; the second as

a percentage of the test compound as measured by dissolved organic carbon (DOC) changes [OECD Guidelines for Testing of Chemicals (Method DGXI 283/82, Revision 5) (13)]; and the third, as a percentage of the test compound as measured by specific substrate analysis.

Graphical representation of percent biodegradation based on the BOD/ThOD ratio were developed against time for each test compound. The experimental DOC data for the initial samples and samples for reaction flasks collected at the end of experimental run were used to calculate the percent biodegradation based on the percent of DOC removal in the culture system.

Figure 1 illustrates a representative oxygen uptake curve for aniline and the endogenous controls. Figure 2 shows the replicate pentaerythritol oxygen uptake curves and the toxicity control (pentaerythritol plus aniline) curve, Figure 3 illustrates a representative graphical treatment of the percent biodegradation of pentaerythritol with time, which was developed for each test compound (OECD studies).

Determination of Kinetic Parameters of Biodegradation

The Monod equation, relating cell growth to biomass and substrate concentration and the linear law, relating cell growth to substrate removal are the most popular kinetic expressions which can provide adequate description of growth behavior during biodegradation of substrate. The Monod relation states that cell growth is first order with respect to biomass concentration (X) and mixed order with respect to substrate concentration (S) by the equation

$$dX/dt = (Su_mX)/(K_s + S) \quad (1)$$

Cell growth is related to substrate removal by the linear law by the equation

$$dX/dt = -Y(dS/dt) \quad (2)$$

The kinetics of biodegradation were evaluated by quantifying μ_m, K_s and Y kinetic parameters expressed in the equation for the rate of substrate removal, r_s:

$$r_s = -\mu_m X/Y \quad (3)$$

where X is the concentration of biomass capable of utilizing the organic substrate and Y is the biomass yield coefficient for the compound and in the Monod equation:

$$\mu = \mu_m S_s/K_s + S_s \quad (4)$$

(if the compound is not inhibitory to its own biodegradation) or by the Haldane equation:

$$\mu = \mu_m S_s/K_s + S_s + (S_s^2/K_I) \quad (5)$$

if the compound is inhibitory. In these equations, μ_m is the maximum specific growth rate, K_s is the half saturation coefficient, K_I is the inhibition coefficient and S is the concentration of substrate.

Determination of Rates of Exponential and Declining Growth.

The first order kinetic rate constants (specific growth rate parameters) were determined by the linearization of the BOD curves or transforming the typical BOD curve to the linear function of time t, by the relationship of log dO_u/dt to t, which gives straight lines expressing the exponential and declining endogenous phases of the BOD curve as shown in Figures 4 and 5. The slope of the Ln(d oxygen uptake/dt) versus t give specific rate constants of the exponential growth phase (μ values) and the declining growth phase (μ' values) of the BOD curve as described by Dojlido,[1] Tabak *et al.*,[3] Oshima *et al.*,[4] and Tabak *et al.*[14,15]

Acclimation time values (t_0) and the time values for the initiation and termination of the declining growth phase (t_1 and t_2) for each test compound were determined from linearized expressions of BOD curves.

Estimation of Monod Kinetic Parameters.

The estimations of the Monod Kinetic parameters, maximum specific growth rate constant, μ_m, half saturation constant, K_s and growth yield constant, Y were determined directly from experimental oxygen uptake curves without the consideration of initial growth and growth yield assumption (Jobbagy, Grady and Tabak[16] and Tabak *et al.*[15]

If the concentrations of the substrate, the products and the biomass are all expressed in BOD units, then the oxygen uptake (O_u at any time in the batch reactor may be calculated from

$$O_u = (S_{so} - S_s) - (X - X_o) - (S_p - S_{po}) \qquad (6)$$

where S_{so}, S_{po} and X_o are the concentrations of substrate, products and cells, respectively, at time zero, and S_s and S_p are the concentrations of substrate and product, respectively, at time t.

To apply equation (4) for the determination of kinetic coefficients, equations must be available which express the concentrations of soluble substrate (S_s), soluble product (S_p) and biomass (X) as functions of time. For batch reactors, those equations are:

$$dS_s/dt = -(\mu_m/Y)S_sX/K_s + S_s) \qquad (7)$$

$$dS_p/dt = (Y_p\mu_m/Y)S_sX/(K_s + S_s) \qquad (8)$$

$$dX/dt = \mu_mS_sX/(K_s + S_s) - K_sbX/(K_s + S_s) \qquad (9)$$

where S_s = soluble substrate concentration; S_p = soluble product concentration; Y_p = product yield; and b = decay coefficient.

To calculate oxygen uptake in a batch reactor, equations (7), (8) and (9) must be solved simultaneously, and the resulting values of S_s, S_p and X over time are substituted into equation (6).

Determination of Y Constant.

Y - the true yield parameter or the ratio of growth of biomass to substrate utilization, can be obtained from the experimental oxygen uptake curve at the initiation of the plateau of the curve as shown in Figure 6, with the use of equation:

$$Y = (1-O_{upt}/S_o)-Y_p \qquad (10)$$

Where O_{upt} is the cumulative oxygen update value at the initiation of plateau, S_o is the concentration of substrate at time zero, and Y_p is the product yield.

A vertical line is drawn at the point of intersection of the tangents of the exponential and plateau phases of the curve. The oxygen uptake value obtained at the point of intersection of the vertical line (drawn through intersection of tangents) and oxygen uptake curve is the O_{upt} value - [cumulative oxygen uptake value at the initiation of plateau].

The O_{upt} value is then substituted into equation (10) , for Y determination. Y_p is soluble product concentration formed, divided by initial substrate concentration. In this study, product yield (Y_p) was negligible.

Determination of μ_m Constant.

The initial estimate of μ_m is obtained by the technique of Gaudy *et al*.[5,6] If Y_p is assumed to be zero, equation (8) is eliminated and if b (decay constant) is assumed to be zero, equation (9) is simplified to

$$dX/dt = (\mu_m S_s X)/(K_s + S_s) \qquad (11)$$

Combining equation (7) and (11) and integrating from S_{so} to S_s and from X_o to X gives:

$$X = X_o + Y(S_{so} - S_s) \qquad (12)$$

If the assumption that $S >> K_s$, the term $S_s/K_s + S_s$ in equations (7) and (11) or (1) approaches one, and these systems can be simplified through the use of equation (12) to give equation

$$dX/dt = \mu_m X \qquad (13)$$

Integrating equation (13) and combining with equation (2) and than substituting in equation (6) with P and P_o both equal to zero gives

$$Ln[X_o + O_u/(1/Y)-1] = Ln(X_o) + \mu_{m(est)} \quad (14)$$

The plot of $Ln[(X_o + O_u)/((1/Y) - 1)]$ versus time will give a straight line with slope μ_m. The accuracy of μ_m(est) will depend upon the size of K_s, but this value is good enough as an estimate.

μ_m - the maximum specific growth rate can be determined from experimental oxygen uptake curve plot in the following manner:

(1) Values of the change of O_u with time (dO_u/dt) or slopes are determined along the entire experimental oxygen uptake curve as shown in Figure 6.

(2) These dO_u/dt (slope) values are then plotted against the cumulative O_u values for each time interval, as shown in Figure 7.

(3) The slope of the developed linearized form of oxygen uptake curve is the estimated μ_m value.

Determination of K_s Constant.

K_s - the half saturation constant or the substrate concentration at which the specific growth rate is 1/2 the maximum specific growth rate can be obtained from the experimental oxygen uptake curve in the following manner:

(1) Value of O_{ut} can be calculated from the plot of (dO_u/dt) versus O_u provided the K_s value is 1 or less (insignificant in comparison to S_o value) and the plot contains a linear section with the slope μ_m, as shown in Figure 8.

(2) Other (dO_u/dt) versus O_u plot in which the slope deviates from μ_m because of larger K_s values (more significant in comparison to S_o) is illustrated in Figure 9.

(3) The value of dO_u/dt is determined at the intercept of the straight line developed from the plot of dO_u/dt versus O_u (Figure 8) which contains a linear section with slope μ_m.

(4) Beginning with the value of 1/2 the intercept value, another straight line (b) is constructed with the slope 1/2 that of the slope of original line (a) whose slope is μ_m.

(5) At the point where line (b) intercepts the declining experimental curve of the plot, a vertical line from that point of interception can provide the value of O_{ut} on the x axis.

Integrating equation (13) and combining with equation (2) and than substituting in equation (6) with P and P_o both equal to zero gives

$$Ln[X_o + O_u/(1/Y)-1] = Ln(X_o) + \mu_{m(est)} \quad (14)$$

The plot of $Ln[(X_o + O_u)/((1/Y) - 1)]$ versus time will give a straight line with slope μ_m. The accuracy of μ_m(est) will depend upon the size of K_s, but this value is good enough as an estimate.

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(5) At the point where line (b) intercepts the declining experimental curve of the plot, a vertical line from that point of interception can provide the value of O_{ut} on the x axis.

(6) This O_{ut} value is then used in the determination of K_s with the use of the equation

$$S_t = S_o - O_{ut}/(1-Y_p-Y) = K_s \quad (15)$$

where S_o = initial substrate concentration and S_t = substrate concentration at time t.

(7) When the O_{ut}, Y, Y_p, and S_o values are plugged into the equation, the value of S_t can be calculated - which is the value of K_s (in systems where K_s value is 1 or less).

Thus the oxygen uptake value O_{ut} associated with 1/2 of the estimate of u_m is used in $K_s = S_o - O_{ut}/(1-Y_p-Y)$ to get the estimate of K_s. The major impact of K_s is upon the shape of the oxygen uptake curve in the region of the plateau. Comparison of the experimental curves to a family of standardized curves as an initial estimate provides an initial estimate of K_s that is sufficient for non-linear curve fitting techniques for quantitation of the kinetic parameters.

Modifications for Volatile and Sparingly Soluble Compounds

Volatile Compounds--

The equations 7-9 are valid for a non-volatile compound present at a concentration below its solubility limit, so they have to be modified for volatile and sparingly soluble compounds. The respirometer reactors being sealed, when compound is added it partitions itself between gas and liquid phases. As biodegradation proceeds and the substrate concentration decreases in liquid phase, it is transferred from the gas phase to the liquid phase to maintain equilibrium in accordance with Henry's law. If S is the total substrate concentration in the system, S_L is the substrate concentration in the liquid phase, and V_g and V_L are volumes of gas and liquid phases respectively, then

$$S_L = S/(1 + HV_g/V_L) \quad (16)$$

where H is the dimensionless Henry's Law Constant, specified by the following equation

$$H = \frac{[\text{Henry's Law Constant in atm/moles/m}^3]}{RT} \quad (17)$$

R is the universal gas constant and T is the temperature in °K.

Equation 16 was derived to quantify the partitioning between the gas and liquid phases. The use of Henry's Law Constant is justified since the molar concentration of the compound is approximately 10^{-4}, and hence the activity coefficient in the liquid phase is equal to 1.0.

Let S_{L1} = concentration of substrate in liquid phase at time t_1

S_{L2} = concentration of substrate in liquid phase at time t_2

then using equation (7)

$$S_{L2} = S_{L1} - [(\mu_m S_{L1} X_{t1}/Y)dt/(K_s + S_{L1})] + (S_{L1} - S_{L2})(HV_g/V_L)$$

As dt tends to zero the above equation results in

$$dS_L/dt = - (\mu_m S_L X/Y)/[(K_s + S_L)(1 + HV_g/V_L)] \quad (18)$$

Substituting equation (16) in equation (18)

$$dS/dt = - (\mu_m SX/Y)/(K_s' + S) \quad (19)$$

where $K_s' = K_s(1 + HV_g/V_L)$. Similarly equations (8) and (9) change to

$$dS_p/dt = (Y_p \mu_m SX/Y)/(K_s' + S) \quad (20)$$

$$dX/dt = [(\mu_m SX)/(K_s' + S)] - [(K_s' bX)/(K_s' + S)] \quad (21)$$

A sparingly soluble compound will dissolve into the solution phase up to its solubility limit and the undissolved part will exist as a different phase. As the compound undergoes biodegradation in solution phase more of the undissolved part dissolves into the solution, keeping its concentration in solution phase constant at the solubility limit. Therefore, degradation proceeds at a constant substrate concentration as long as total substrate concentration in the system is greater than its solubility limit. Now, if the compound is also volatile then depending on Henry's law constant it will partition itself into the gas phase and liquid phase. When the microorganisms degrade this compound from the solution phase, then compound from the undissolved part dissolves into solution phase. This keeps the compound concentration in the solution phase and gas phase constant. If S is the total substrate concentration (per unit volume of liquid phase) in the system and S_s is its solubility limit then the concentration in solution phase and gas phase will be constant as long as $S > S_s/(1 + HV_g/V_L)$. So if substrate concentration is held constant at S_s in equations (7), (8) and (9) then they can be solved analytically and the solutions are

$$X = X_o \exp[(\mu_m S_s - bK_s)t/(K_s + S_s)] \quad (22)$$

$$S = S_o - [(\mu_m S_s/Y)/(\mu_m S_s - bK_s)](X - X_o) \quad (23)$$

$$S_p = [(Y_p \mu_m S_s/Y)/(\mu_m S_s - bK_s)](X - X_o) \quad (24)$$

Thus, equations (22), (23) and (24) should be used when the total concentration of the substrate, S is greater than $S_s(1 + HV_g/V_L)$ and equations (19), (20) and (21) should be used when the total substrate concentration, S is less than or equal to $S_s(1 + HV_g/V_L)$.

Quantitation of Monod Kinetic Parameters.

The methodology for quantitation of the Monod kinetic parameters requires the use of the above specific methods for estimating them initially and subsequently followed by computer simulation methods coupled with non-linear curve fitting techniques and is based on the use of measured values of initial growth and growth yield. The method requires the use of the kinetic equation relating growth rate of biomass in presence of substrate, the substrate utilization rate, product formation rate and rate of oxygen consumption from O_2 uptake (BOD) curves to calculate and use the theoretical oxygen consumption data to quantitate the biokinetic parameters.

The determination of the kinetic parameters associated with biodegradation requires a series of steps. The initial substrate (S_{so}) and biomass (X_o) concentration must be carefully measured in COD units. The ratio of the two values must lie in a certain range in order to allow independent evaluation of μ_m, K_s and Y (Simkins and Alexander[17,18]). Grady's studies[7,8] have shown that a S_{so}/X_o ratio of around 20 works well. The value of Y_p may be estimated by determining the residual stable SCOD concentration after substrate depletion (plateau area). It is numerically equal to the residual SCOD divided by the initial SCOD. The value of the decay coefficient, b, may be determined by fitting to the oxygen consumption curve after the plateau when the only activity contributing to oxygen consumption is endogenous metabolism and cell decay. Once X_o, S_{so}, Y_p and b are known, μ_m, K_s and Y may be determined by non-linear curve fitting techniques (Grady[7,8]).

The technique involves the calculation of a theoretical oxygen consumption using oxygen uptake equation (4) and equations for substrate, product and biomass concentrations (5) - (7) with assumed Monod parameters. The residual sum of squared errors (RSSE) associated with the difference in calculated and experimental oxygen uptake values is used to obtain new estimates. The above procedure is repeated until a minimum RSSE is found.

The Grid Search technique was selected as a most suitable non-linear curve fitting technique for application in the determination of the kinetic parameters from oxygen uptake data, because it can allow easy discrimination between local minimal and the global minimum RSSE. This technique enables a comparison between the calculated and experimental oxygen uptake data. The value of Y is fixed. For this value of Y, a pair of μ_m and K_s which give RSSE is found on a μ_m:K_s plane. The above procedure is repeated with other values of Y. Values of μ_m, K_s and Y which give minimum RSSE associated with the difference in calculated and experimental oxygen uptake data constitute the best values of the kinetic parameters.

The values of μ_m, K_s and Y developed from grid search technique, which when substituted into equations 5-7, will provide X and S values, which (when substituted into oxygen uptake equation 4) will in turn provide calculated oxygen uptake values at the region of the plateau, closest to the experimental oxygen uptake values, with a minimum RSSE, will constitute the best quantitative kinetic parameter values.

Development of Multi-Level Respirometric Biodegradation Testing Protocol

The oxygen consumption data generated with the use of electrolytic respirometry have been adequately utilized for assessing the biodegradative activity of sludge microbiota, the biodegradability/toxicity of toxic organic compounds, as well as for the determination of the intrinsic kinetic parameters of biodegradation. Methodologies have been developed for quantitating biodegradability and biodegradation kinetics of representative classes of RCRA toxic organics, with the resultant development of a comprehensive multi-level respirometric biodegradation testing protocol based on oxygen consumption data.

The methods of each successive testing level of the respirometric protocol are characterized by increased complexity and a consequent higher cost for performing the tests pertaining to each testing level. Accordingly, the testing levels can be selected as appropriate to the research needs.

Studies to assess the biodegradability and biodegradation rates of toxic organics by sludge microbiota with the use of respirometric oxygen uptake data can involve the use of one of more levels of the protocol, depending on the amount of information needed for assessing the biodegradability of the organic toxic pollutant or the toxicant bearing waste for determination of its fate and rate of biotreatment in the municipal or industrial waste treatment systems. A logic flow diagram of the respirometric biodegradation testing protocol, providing a brief description of each of the testing levels, is shown in Figure 10.

RESULTS AND DISCUSSION

Respirometric biodegradability, biokinetic and Monod kinetic data for selected RCRA alkyl benzenes and phenols are reported in this paper. The electrolytic respirometry oxygen uptake data for the test compounds, the control reference compound aniline, the inhibition and endogenous control systems were generated revealing the lag phase (acclimation phase), the biodegradation (exponential) phase, the different bio-reaction rate slopes (characteristic of the test compound) as well as the plateau region at which the biooxidation rate reaches that of the endogenous rate of microbial activity.

Based on the biokinetic equations relating growth rate of microbiota in presence of above compounds, the substrate utilization rate, and rate of oxygen uptake (BOD) curves, specific growth rate kinetic parameters (biodegradation rate constants) were derived as slope values of the linearized plots (plots of the log of DO_u/dt) of exponential and declining growth phases of the BOD curve. The acclimation time values (t_0), and time values for the initiation and the termination of the declining growth phases (t_1 and t_2) for the test compounds and aniline were also generated.

The estimations of the Monod kinetic parameters for benzene, phenol, phthalate, and ketone compounds were determined directly from experimental

oxygen uptake curves without the consideration of initial growth and growth yield assumption.

The biodegradation of benzene, toluene, ethyl benzene, m- and p-xylenes, tert-butyl benzene, sec-butyl benzene, butyl benzene, cumene, 1-phenyl benzene and the reference compound, aniline at 100 mg/L concentration by 30 mg/L sludge biomass (as measured by oxygen consumption by sludge microbiota in mg O_2/L) was followed over a period of 20 days. The electrolytic respirometry oxygen uptake and BOD curves were generated and graphical treatment of the percent biodegradation was established for each compound. Figure 11 demonstrates typical oxygen uptake and BOD curves for p-xylene and p-xylene + aniline and Figure 12 illustrates graphically the % biodegradation of p-xylene with time.

The percent biodegradation data based on the BOD/ThOD ratios for benzene, toluene, ethyl benzene, m- and p-xylene and the reference compound, aniline, are summarized in Table 1. All of the above alkyl benzene compounds were shown to be biodegradable substrates at concentration levels of 100 mg/L when exposed to 30 mg/L of activated sludge biomass under the environmental conditions of the respirometric testing procedure, and within the period of 20 days of incubation.

The toxicity test control flask respirometric data revealed no inhibitory effects by these test compounds at the 100 mg/L concentration levels on the bio-oxidation of aniline by sludge microbiota.

Table 2 summarizes the bio-kinetic data for the benzenes studied, showing the specific growth rate constants for the exponential growth phase (μ values) and for the declining growth phase (μ' values) of the linearized form of the BOD curves of these compounds, as well as the t_0, t_1 and t_2 kinetic parameters. Figure 13 shows a typical plot of $Ln(dO_u/dt)$ vs. time for toluene, from which the kinetic parameters were determined.

Table 3 summarizes the Monod kinetic parameter (μ_m, K_s, Y_g) data for these benzene compounds.

CONCLUSIONS

The experimental data of respirometric studies with several classes of organic compounds definitely demonstrate that it is possible to measure the biodegradability (percent biodegradation - as a ratio of BOD to ThOD) and to determine the kinetics of degradation of single organic compounds by using only measurements of oxygen consumption in respirometric batch reactors. The values of the kinetic parameters determined from oxygen consumption data were demonstrated to be similar to those based on the measurements of substrate removal and those made with cell growth data.

Studies are currently in progress to determine the effect of temperature, and different sources of sludge biomass (domestic and industrial wastewater treatment) on the biodegradation kinetics derived from electrolytic respirometry.

ACKNOWLEDGEMENTS

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TABLE 1. SUMMARY OF RESPIROMETRIC BIODEGRADATION DATA FOR SELECTED BENZENES

PERCENT BIODEGRADATION (BASED ON % BOD REMOVAL)

Time (days)	Aniline	Benzene	Toluene	Ethylbenzene	o-Xylene	p-xylene
0.0	0.0	0.0	0.5	0.0	0.0	0.0
1.0	2.58	2.11	2.78	2.27	1.35	1.58
2.0	3.43	2.11	8.81	2.27	2.14	1.58
3.0	3.94	4.15	85.11	2.99	69.68	1.58
4.0	14.95	4.97	89.04	3.78	69.68	9.4
5.0	102.0	71.5	94.34	4.29	76.68	70.38
6.0	102.0	74.22	97.92	40.25	82.58	82.68
7.0	102.0	81.85	100.0	73.91	84.67	95.58
8.0	102.0	93.37	101.18	73.78	87.03	99.49
9.0	102.0	93.89	103.48	83.94	87.79	101.76
10.0	102.0	95.45	103.68	88.86	90.12	104.7
11.0	102.0	95.45	103.48	88.86	90.15	105.78
12.0	102.0	96.13	103.48	89.65	90.97	106.7
13.0	102.0	97.46	103.48	91.00	91.80	108.6

TABLE 2. SUMMARY OF BIO-KINETIC DATA FOR SELECTED BENZENE COMPOUNDS

COMPOUNDS	THOD for 100 mg	t_0 (days)	t_1 (days)	t_2 (days)	μ (day-1)	μ' (day-1)
Aniline						
(Experiment 1)	310	4.00	4.70	4.83	2.78	3.29
(Experiment 2)	310	4.00	4.65	4.79	3.00	8.68
Benzene	308	4.50	4.87	5.00	8.57	25.30
Ethyl benzene	317	4.00	4.21	4.83	8.33	44.00
Toluene	313	2.00	2.20	2.42	8.75	14.93
p-Xylene	317	3.90	4.22	4.83	9.94	6.50
o-Xylene	317	2.00	2.35	2.50	6.60	29.00
tert-Butyl benzene	322	4.40	5.12	5.70	1.21	1.09
sec-Butyl benzene	322	3.50	4.00	5.70	0.78	0.73
Cumene	320	2.40	2.79	3.00	2.31	2.24
Butyl benzene	322	3.30	3.92	4.56	2.42	2.42
1-Phenyl hexane	326	4.00	4.55	5.15	1.05	1.90

μ = specific growth rate constant for exponential growth phase of BOD curve.

μ' = specific growth rate constant for declining growth phase of BOD curve.

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TABLE 3. SUMMARY OF MONOD KINETIC PARAMETER DATA FOR SELECTED BENZENE COMPOUNDS

COMPOUNDS	Lag Time (t_o) days	Y, mg biomass / mg substrate	μ_m (day-1)	K_s mg/l
Aniline	4.00	0.38	6.15	6.10
Benzene	4.50	0.23	9.30	22.16
Ethyl benzene	4.00	0.23	9.00	11.01
Toluene	2.00	0.20	9.09	56.74
p-Xylene	3.90	0.26	8.33	97.00
m-Xylene	2.00	0.14	11.50	35.61
tert-Butyl benzene	4.40	0.66	1.80	69.04
sec-Butyl benzene	3.50	0.64	2.39	22.00
Cumene	2.44	0.66	2.44	87.64
Butyl benzene	3.30	0.80	5.17	34.50
1-Phenyl hexane	4.00	0.67	2.15	35.09

μ = maximum specific growth rate.

K_s = half saturation constant; concentration of substrate at $\mu_m/2$.

Y_g = growth yield, mg biomass formed/mg substrate consumed.

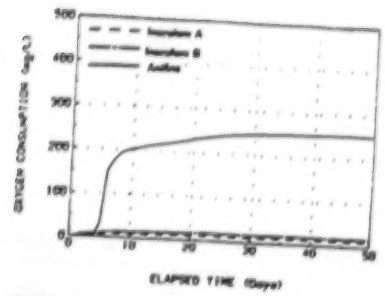

FIGURE 1 OXYGEN UPTAKE DATA ON ANILINE

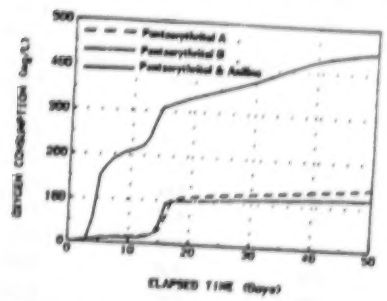

FIGURE 2 BIOLOGICAL OXYGEN UPTAKE CURVE
(Run 1 Sample No. 90-12)

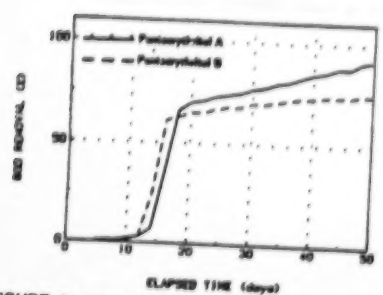

FIGURE 3 BIODEGRATION (% BOD REMOVAL) CURVE
(PENTAERYTHRITOL)

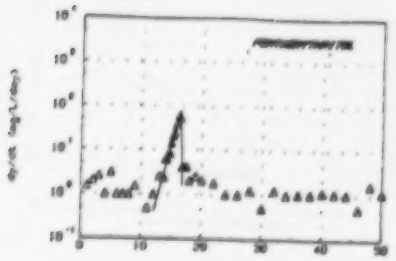

FIGURE 4 Log (dy/dt) vs t Plot
(PENTAERYTHRITOL)

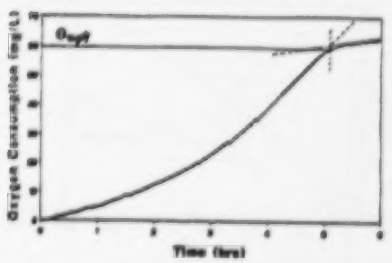

FIGURE 5 DETERMINATION OF THE START OF THE PLATEAU IN OXYGEN CONSUMPTION FOR USE IN ESTIMATING Y_g

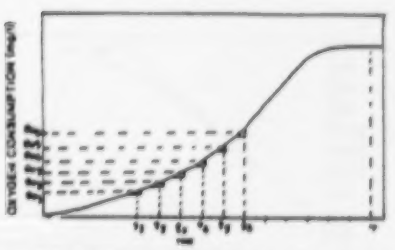

FIGURE 6 PLOT OF OXYGEN UPTAKE RATE
(Ou VERSUS TIME)

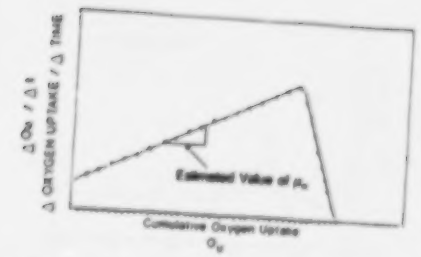

FIGURE 7 PLOT OF $\Delta O_u / \Delta t$ VALUES OR SLOPES VERSUS THE CUMULATIVE O_u VALUES

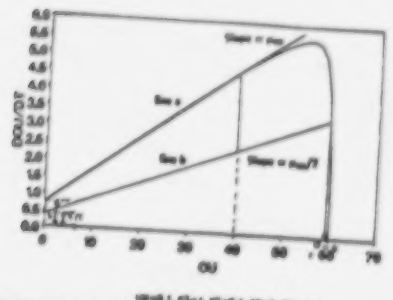

FIGURE 8 Plot showing $(P\,Ou/t\,t)$ versus Ou_t curve containing a linear section with a slope $= \rho_o$ to be used in estimating the values of K_{-1}/t cases where K_{-1} values are negligible in comparison to S_0.

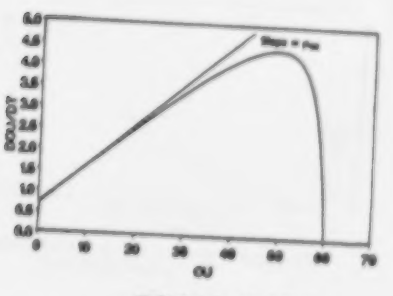

FIGURE 9 Plot of (dOu/dt) versus Ou_t in which the slope deviates from ρ_o because K_{-1} values are more significant in comparison to S_0. ($P_{-1} = 5$)

FIGURE 10 LOGIC FLOW DIAGRAM FOR THE RESPIROMETRIC BIODEGRADATION TESTING PROTOCOL

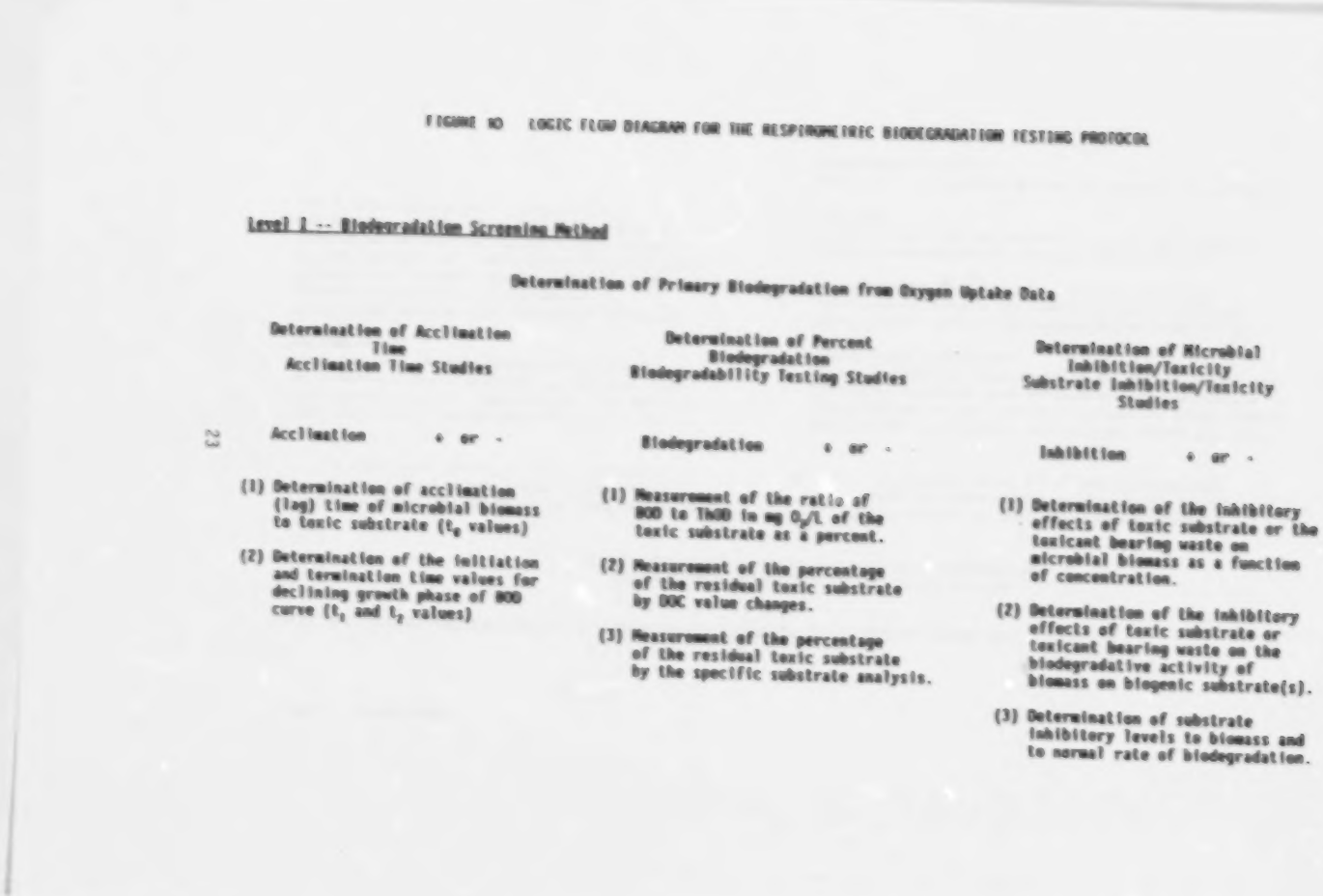
Level I -- Biodegradation Screening Method
Determination of Primary Biodegradation from Oxygen Uptake Data
Determination of Acclimation Time
Acclimation Time Studies
Determination of Percent Biodegradation
Biodegradability Testing Studies
Determination of Microbial Inhibition/Toxicity
Substrate Inhibition/Toxicity Studies
Acclimation + or -
Biodegradation + or -
Inhibition + or -
(1) Determination of acclimation (lag) time of microbial biomass to toxic substrate (t_0 values)
(2) Determination of the initiation and termination time values for declining growth phase of BOD curve (t_1 and t_2 values)
(1) Measurement of the ratio of BOD to ThOD in mg O_2/L of the toxic substrate as a percent.
(2) Measurement of the percentage of the residual toxic substrate by DOC value changes.
(3) Measurement of the percentage of the residual toxic substrate by the specific substrate analysis.
(1) Determination of the inhibitory effects of toxic substrate or the toxicant bearing waste on microbial biomass as a function of concentration.
(2) Determination of the inhibitory effects of toxic substrate or toxicant bearing waste on the biodegradative activity of biomass on biogenic substrate(s).
(3) Determination of substrate inhibitory levels to biomass and to normal rate of biodegradation.

FIGURE 10 (continued)

Level II -- Studies of Biokinetics of Biodegradation Based on Respirometric Oxygen Uptake Data

Determination of Specific Growth Rates and the Initial Estimates of Monod Kinetics of Biodegradation

Determination of the Rates of Exponential and Declining Growth Phase of BOD Curve Determination of μ and μ' Constants

Application of O_2 uptake data and BOD values to the determination of BOD kinetics. Utilizaiton of biokinetic data (exponential and declining growth rates) for biodegradation efficiency determination.

First order kinetic rate contstants (specific growth rate parameters) determined by linearization of BOD curves through a relationship of log (dOu/dt) to t which gives straight lines expressing exponential and declining phases of BOD curves. Slopes of these lines represent μ and μ' parameter values respectively.

Determination of the Monod Kinetic Parameters from the Experimental Oxygen Uptake Curves Determination of μ_m, K_s and Y Parameters

Application of O_2 uptake data and BOD values for the determination of the initial estimates of Monod kinetic parameters directly from experimental O_2 consumption curves without the consideration of initial growth and growth yield assumption.

These initial kinetic parameters estimate data can be successfully applied for biodegradation efficiency determination in wastewater treatment systems.

Specific methodologies have been developed for obtaining the initial estimates of the Monod kinetic parameters.

Use of pre-accclimated biomass or biomass acclimated in respirometric vessels.

FIGURE 10 (continued)

Level III -- Studies to Quantitate Monod Kinetic Parameters from Respirometric Oxygen Uptake Data

Use of Initial Estimates of Kinetic Parameters, μ_m, K_s and Y, to Quantitate these by Means of Computer Simulation Methods Coupled with Non-Linear Curve Fitting Techniques

Methodology based on the kinetic relationship between growth rate, oxygen uptake rate and toxic substrate concentration in a mass balance equation to develop intrinsic kinetic parameter data for prediction of the fate of the substrate in full scale treatment systems

Methodology supported by Proof-of-Concept Data which show an agreement between the values of the kinetic parameters obtained from O_2 consumption data and those obtained from traditional measurement of substrate removal (BOC, SCOD, ^{14}C) or cell growth.

Methodology requires the use of kinetic equations relating growth rate in presence of toxic substrate, substrate utilization rate, soluble product formation rate and rate of oxygen uptake in order to calculate and use the theoretical O_2 consumption data (O_2 uptake curves) to quantitate the biokinetic parameters.

Methodology is based on the use of measured values of initial growth (X_o) (biomass concentration), substrate concentration (S_{wo}) and growth yield (Y), product yield (Y_p) (determined by fitting O_2 uptake curve after the pilferage (O_2 consumption due to endogenous metabolism and cell decay).

Once X_o, S_{wo}, and Y_p and b (cell decay) values are known, the kinetic parameters, μ_m, K_s and Y are quantitated by the non-linear curve fitting techniques. Initial kinetic parameter estimates are used in the method.

The non-linear curve fitting technique (Grid Search Method) was shown to be most applicable, since it allows easy discrimination between the calculated and experimental O_2 uptake data. RSSE associated with differences in calculated and experimental O_2 uptake values are used to obtain new estimates of kinetic parameters. This procedure is repeated till minimum RSSE is formed.

Grid Search Technique - Value of Y is fixed. For this value of Y, a pair of μ_m and K_s values which give RSSE is found on a μ_m-K_s plane. The above procedure is repeated with other values of Y. Values of μ_m, K_s and Y which give minimum RSSE associated with the difference in calculated and experimental O_2 uptake data constitute the best values for the kinetic parameters.

Level III uses either pre-acclimated biomass or biomass acclimated in respirometric vessels.

NOTE TO EDITORS

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FIGURE 10 (continued

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Level IV -- Studies to Determine the Extent of Biodegradation

Tests for Assessment of Ultimate Biodegradability during Respirometric Experiments

Radioisotope Respirometry Electrolytic Respirometry Coupled with Radioisotope Technology	Respirometry Coupled with Specific Substrate Analysis
Use of ^{14}C labeled toxic substrates. Trap $^{14}CO_2$ and measure ^{14}C intermediate metabolites during incubation. Measure radioactivity of the liquid and gaseous phases of the contents in the respirometric vessel system by scintillation counter techniques.	Analysis of the residual parent toxic substrate, intermediate metabolites and end products of metabolism by GC, GC/MS and HPLC technology.
Liquid and gaseous samples for the radioactivity analyses can be taken intermittently during the respirometric run by means of specially adapted syringes from side arms of the respirometric vessel.	Liquid and gaseous samples of the culture system can be manually taken from side arms of the respirometric vessel with specially adapted syringes during the respirometric run.
	Automated analysis of the culture system can be performed by way of specially designed system coupling the respirometric vessels to the analytical instruments.

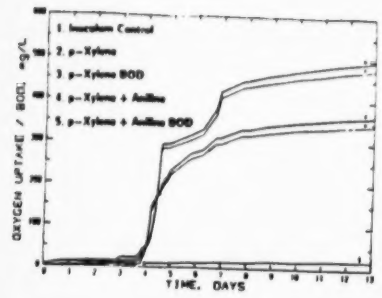

FIGURE 11 BIOCHEMICAL OXYGEN UPTAKE AND BOD DATA FOR P-XYLENE AND P-XYLENE + ANILINE

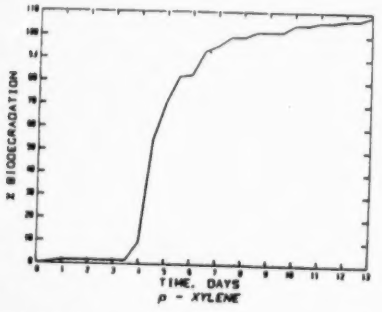

FIGURE 12 BIODEGRADATION DATA FOR P-XYLENE

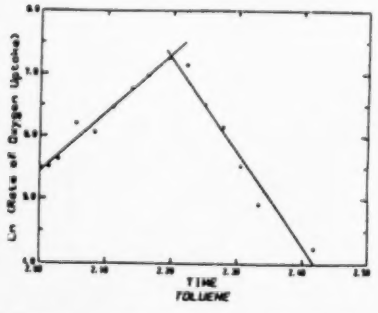

FIGURE 13 PLOT OF LOG ($\Delta Ow/\Delta t$) FOR TOLUENE